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Award Number: DAMD17-00-1-0162

TITLE: Compartmentalized Signaling and Breast Cancer Cell Proliferation

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REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Compartmentalized Signaling and Breast Cancer Cell Proliferation

5. FUNDING NUMBERS
DAMD17-00-1-0162

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

This proposal will test a unique hypothesis that Src-family kinases direct the ErbB receptor traffic into an intracellular compartment where proliferation signals are generated. This hypothesis is based on observations that while Cbl proto-oncoprotein facilitates the down-regulation of ErbB receptors from cell surface and functions as a negative regulator, Src tyrosine kinase enhances paradoxically enhance both the internalization of EGFR and the EGFR-mediated mitogenic signals. The proposed studies will test this hypothesis using mammary epithelial cells made to overexpress EGFR or ErbB2 together with Src. The insights gained from this model system will be directly relevant to a large proportion of breast cancers where ErbB receptors and Src-family kinases are co-overexpressed. Validation of our hypothesis will represent a shift in the current paradigm of normal and aberrant ErbB receptor signaling and may provide novel targets for therapeutic intervention relevant to ErbB-overexpressing breast cancers, which carry a significantly worse prognosis and are frequently hormone-unresponsive.

14. SUBJECT TERMS
Breast Cancer, ErbB receptors, signal transduction, tyrosine kinases

15. NUMBER OF PAGES
23

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction:

The experiments proposed in the application submitted for funding were designed to test a unique hypothesis that Src-family kinases direct the ErbB receptor traffic into the recycling endosomal compartment, where proliferation signals are generated from the internalized pool of activated ErbB receptor tyrosine kinases. This hypothesis was based on previous observations that the potency of mitogenic signals emanating from ErbB receptors correlates directly with the ability of these receptors to avoid the lysosomal pathway of degradation and to be sorted into the recycling pathway instead. For example, ErbB1 (EGFR) is targeted to the lysosomal as well as recycling pathways, whereas the more potent ErbB2 receptor is primarily recycled. Work in our laboratory has defined the Cbl proto-oncoprotein as a novel regulator of this process. Cbl enhances the delivery of ErbB1 into the lysosomal pathway. In contrast, Cbl does not interact with ErbB2, which is primarily recycled. Paradoxically, Src tyrosine kinase enhances the EGFR internalization while at the same time enhancing EGFR-mediated mitogenic signals. This led us to hypothesize that Src directs ErbB receptors away from lysosomes and into a recycling endosomal compartment where these receptors continue to transmit proliferation signals. The proposed studies are designed to test this hypothesis using mammary epithelial cells made to overexpress EGFR or ErbB2 together with Src. This model system will provide insights directly relevant to a large proportion of breast cancers where ErbB receptors and Src-family kinases are co-overexpressed. Validation of our hypothesis will represent a shift in the current paradigm of normal and aberrant ErbB receptor signaling and may provide novel targets for therapeutic intervention relevant to ErbB-overexpressing breast cancers, which carry a significantly worse prognosis and are frequently hormone-unresponsive.

Minor Modifications to Statement of Work:

The overall goals and strategies of the project remain unchanged. However, in the original proposal we wished to test our novel hypotheses in the context of the EGFR (which is efficiently targeted for degradation and therefore less potent at signaling) and ErbB2 (which is inefficiently targeted for degradation and more potent at signaling), using the mammary epithelial cell line 16A5 developed by our collaborator Dr. Vimla Band. Work from their laboratory and other recently published work, however, raised the possibility that use of 16A5 cells for our studies could be problematic. Therefore, we have carried out an extensive set of studies to determine if 16A5 is suitable for our analyses and to identify alternate mammary epithelial cells (see below). These studies have identified a hTERT-immortalized human mammary epithelial cell line 76N-TERT, derived from the same parental strain as 16A5, as an alternative line for our studies. As a result of the inclusion of this line, which is neomycin-resistant, we have also had to re-derive our various constructs using appropriate drug-resistance markers, including construction of a new vector. These studies have been added as Tasks 1 and 2 (now completed) to the statement of work, and the timetable of the originally proposed tasks has been modified.

Modified Statement of Work

Task 1. Determine the characteristics of available mammary epithelial cell lines to establish their suitability for studying ErbB receptor recycling endosome pathway (months 1-12).

a. Screen available mammary epithelial cell lines for levels of expression of EGFR.

b. Compare HPV-oncogene immortalized 16A5 cells and hTERT-immortalized 76N-TERT cells for EGF-induced EGFR stimulation.
c. Compare 16A5 and 76N-TERT cells for EGF-induced downregulation of EGFR.

Task 2. Construct pMSCV-based retroviral constructs directing the expression of EGFR, ErbB2 and Src (months 1-12).

a. Construct a new pMSCV vector coding for blasticidin resistance.
b. Clone wildtype and Cbl-independent EGFR and ErbB2, and wildtype Src, into retroviral vectors.
c. Establish the ability of retroviral constructs to direct the expression of expected proteins by transient transfection in 293T cells.

Task 3. Establish mammary epithelial cell transfectants that overexpress EGFR, ErbB2, Src or their combinations (months 12-18).

a. Establish stable clonal transfectant lines of 76N-TERT cells overexpressing the following: EGFR; ErbB2; Src; EGFR plus Src; and ErbB2 plus Src.
b. Establish level of overexpression of exogenous proteins by immunoblotting of whole cell lysates and immunoprecipitates.
c. Quantify relative cell surface levels of EGFR and ErbB2 by flow cytometry with specific antibodies.

Task 4. Determine the impact of overexpressing Src on the mitogenic response and anchorage-independent growth of MECs to ErbB ligands (months 12-24).

a. Measure mitogenic responses to various ligands (EGF, TGFa, HRGb, and an agonistic anti-ErbB2 monoclonal antibody) using 3-H-thymidine incorporation into DNA in MECs overexpressing ErbB receptor and/or Src.
b. Examine if overexpression of Src, ErbB receptors, or their combination leads to anchorage-independent growth, either in the absence or presence of exogenous ligands.

Task 5. Compare the sub-cellular localization of EGFR and ErbB2 in parental versus transfected MECs (months 12-24).

a. Establish if Src overexpression enhances EGFR endocytosis by assaying acid-stable internalization of FITC-labeled EGF, and quantifying the EGFR remaining on the surface after stimulation by flow cytometry.
b. Assess co-localization of the ErbB receptors with Src in the endocytic compartments with two-color conventional and confocal imaging immunofluorescence microscopy, together with immuno-gold electron microscopy.
c. Assess co-localization of ErbB receptors or Src together with markers specific for subcompartments of the endocytic pathway.

Task 6. Determine the impact of interrupting the traffic of the ErbB receptors to and out of recycling endosomes on the distribution of EGFR and ErbB2, and on ligand-induced biological responses (months 24-30).

a. Stably express ARF6-T27N and Rab11-S25N in either parental MECs, or in selected EGFR/Src or Erb2/Src overexpressing MECs using puromycin selection.

b. Examine the localization of EGFR/Erb2 and Src in ARF6-T27N and Rab11-S25N overexpressing MECs and their co-localization with markers of the endocytic pathway.
c. Assess impact of stable expression of ARF6-T27N and Rab11-S25N in EGFR/Src or Erb2/Src overexpressing MECs on mitogenic response to ErbB ligands and anchorage independent growth.

Task 7. Determine the nature of signaling cascades generated by endocytosed ErbB receptors (months 30-36).

a. Prepare plasma membrane and endosomal fractions by sucrose gradient ultra-centrifugation, and compare levels of phosphorylation of ErbB receptors and various substrates, and ErbB receptor association with known signaling proteins in these fractions.
b. Examine selected signaling molecules whose activated and/or receptor-associated pools can be detected by immuno-staining procedures.

Body of Report:

During year one, we have carried out studies that were proposed in the initial proposal only as alternative strategies but became essential in view of recent findings in the laboratory of our collaborator, Dr. Vimla Band, and a report on differing characteristics of EGFR signaling in different mammary epithelial cell lines. These studies now constitute Tasks 1 and 2 of the modified statement of work. Based on these studies, work on Task 3 has been initiated.

Based on our previous studies [1] and additional studies included in the submitted proposal, we had determined that 16A5 mammary epithelial cell line to be suitable for our transfection studies. 16A5 cell line had been derived in Dr. Vimla Band's laboratory from 76N normal mammary epithelial cell strain by immortalizing these cells with HPV16 E6 and E7 oncogenes. Two lines of evidence suggested that exclusive use of this cell line may be problematic and that alternate or additional cell lines would be required. First, Dr. Band's laboratory identified a new HPV16 E6-binding protein, which upon further characterization proved to be Protein Kinase N (PKN), a PKC-related but distinct serine/threonine kinase that is regulated by Rho family of small G-proteins. Since we planned to investigate a recycling pathway of ErbB receptors in these cells, we became concerned that the E6-induced perturbation of PKN in 16A5 cells may lead to aberrant ErbB trafficking given that PKC-related kinases play many roles in receptor signaling and regulation. This concern was further amplified by a recent publication [2] in which PKN (under an alternate name PRK1) was shown to mediate RhoB-induced alterations in trafficking of EGFR at the level of endosomes. This finding provided a rationale for comparison of 16A5 cell line with other non-E6-immortalized mammary epithelial cell lines for EGFR levels, stability and stimulation characteristics. The second line of evidence prompting our reassessment of 16A5 cells came from a recent publication by Burke and Wiley, which for the first time assessed EGFR traffic in untransformed mammary epithelial cell lines that would be comparable to 16A5 cells [3]. The authors showed that 184A1 cell line, thought to represent basal cell phenotype and comparable to 16A5 cells, showed expected ligand-dependent downregulation of EGFR, but also showed an unexpected ligand-independent constitutive EGFR endocytosis, resulting in 30-50% internal pool of EGFR. In contrast, HB2 cell line representing

the luminal cell phenotype was EGF unresponsive and showed a predominantly cell surface EGFR. In view of these findings, we wished to determine the characteristics of available mammary epithelial cell lines to establish their suitability for studying ErbB receptor recycling endosome pathway. We have therefore carried out the following analyses that were indicated as alternatives strategies in Aim 1 of the original proposal but are now listed as Tasks 1 and 2 under modified Statement of Work.

Screening of available mammary epithelial cell lines for levels of expression of ErbB1. We examined the level of EGFR expression on 16A5 and other mammary epithelial lines generated in Dr. Vimla Band's laboratory in comparison with 184A1, a carcinogen-immortalized mammary epithelial cell line used by Burke and Wiley [2]. The cell lines from Dr. Band's laboratory included the following basal cell lines: 76E6, 76E6E7, 16A5 and 76N-TERT derived from 76N cell strain [4]; 70E6 derived from 70N cell strain; 81E6 derived from 81N cell strain; and milk-derived luminal type cell lines M2-E6E7 and M3-E6E7 derived from M2 and M3 milk cell strains respectively. In each case E6 or E7 refers to HPV oncogenes used for immortalization (16A5 is E6/E7-immortalized), whereas TERT represents hTERT catalytic subunit

First, we examined the total EGFR receptor protein expression in various cell lines using anti-EGFR immunoprecipitation followed by immunoblotting (Fig. 1). Notably, the overall EGFR levels in basal cell-type and luminal-type mammary epithelial cell lines derived in Dr. Band's laboratory were comparable although slightly higher in hTERT-immortalized 76N-TERT cells. Notably, EGFR levels in 184A1 cell line were substantially higher. Since Burke and Wiley study reported that the cell surface levels of EGFR on 184A1 and a luminal mammary epithelial cell line were comparable, we also analyzed our cell lines for cell surface EGFR expression using fluorescence-activated cell sorter analysis of live cells stained with an anti-EGFR antibody (Fig. 2). These analyses revealed that all of the cell lines were uniformly EGFR-positive, as expected. Importantly, the cell surface EGFR levels were comparable among the cell lines examined, including the 184A1 cell line, which expressed several-fold higher levels of overall EGFR protein. These findings are consistent with Burke and Wiley's observations that 184A1 and other cells derived from the same parental cell had a substantial intracellular pool of EGFR [3]. Since our examples of basal mammary epithelial cells derived from three separate parental strains (70N, 76N and 81N) revealed comparable levels of EGFR, and all of these were lower than those in 184A1, it is likely that 184-derived cell lines are not a general representation of all basal mammary epithelial cell lines. The fact that 184A1 is a carcinogen-derived cell line suggests the possibility that unknown genetic events could be responsible for its unusual EGFR trafficking pattern reported in Burke and Wiley study. More importantly, our analyses provided a wider array of cell lines to choose from for our analyses.

Since Cbl is an established determinant of EGFR trafficking, and our hypothesis proposed that Src and Cbl play opposite roles in this process, we also quantified the expression of these regulatory proteins in the mammary epithelial cell lines used above. All of the cell lines expressed Src and Cbl, although their levels varied (Fig. 3 and 4). Notably, however, the levels of Cbl were comparable between 16A5, 76N-TERT and 184A1 cell lines. Importantly, the levels of Src were substantially higher in 184A1 cells, which may be related to Burke and Wiley's observation that they carry a substantial intracellular pool of EGFR and would be

consistent with our hypothesis that Src controls the sorting of EGFR to recycling endosomes. Further studies will need to be carried out to determine if this is the case.

Given that the overall as well as cell surface levels of EGFR expressed in 76N-TERT and 16A5 cell lines were comparable, and since 76N-TERT is derived from the same parental cell line as 16A5 but is not HPV-immortalized, we carried out further analyses of these cell lines to determine if 76N-TERT would be a suitable alternative to 16A5 for our studies.

Comparison of HPV-oncogene immortalized 16A5 cells and hTERT-immortalized 76N-TERT cells for EGF-induced EGFR stimulation. As a first test, we examined if 76N-TERT cells show an EGF stimulation pattern comparable to that of 16A5 cells. As a global readout of early activation events, we compared these cells for EGF-induced tyrosine phosphorylation of EGFR and other cellular substrates using anti-pTyr immunoblotting of cell lysates. These analyses indicated that EGF stimulation of 76N-TERT cells induced a robust tyrosine phosphorylation response comparable to that seen in 16A5 cells (Fig. 5). Additional experiments have shown a similar ability of TGF-alpha to stimulate tyrosine phosphorylation of these cells (Fig. 6) and the ability of EGF and TGF-alpha to induce the internalization of EGFR into endosomes (Fig. 7).

Comparison of 16A5 and 76N-TERT cells for EGF-induced down-regulation of EGFR. Given the possibility that HPV16 E6 in 16A5 may influence EGFR trafficking by interacting with PKN, we compared the relative biochemical stability of EGFR after EGF stimulation in 16A5 and 76N-TERT cells (Fig. 8). While the level of EGFR protein decreased substantially in both cases, significant levels of EGFR were still detectable in 16A5 cells 6 hours of EGF treatment. In contrast, EGFR signal was nearly undetectable at 2 hours after EGF stimulation of 76N-TERT cells. Importantly, this effect was specific, as the levels of ErbB2 did not change, as expected. These results support the possibility that HPV16 E6 expression in 16A5 cells may be responsible for the stability of EGFR. Further direct analyses will, however, be required to prove that the observed stability of EGFR in 16A5 cells is due to E6 perturbation of PKN pathway. Since these studies do not directly contribute to the stated goals of this grant, these will not be pursued as part of the present studies. Based on our findings, we have chosen the 76N-TERT cell line as an alternative for our future analyses.

Construction of a new pMSCV vector coding for blasticidin resistance. Use of 76N-TERT cells, which are G418-resistant and prevent us from using Neo-resistance vectors, necessitated the availability of an additional drug marker for co-expression studies. Therefore, we generated and tested a blasticidin-resistance retroviral vector pMSCV-blast to complement the hygromycin (pMSCV-hygr) and puromycin-resistance vector (pMSCV-puro) that are already available. The blasticidin resistance gene cassette was amplified from pEF6/Myc-HisA vector (In-Vitrogen) using the PCR. The PCR primers allowed the incorporation of PshAI and HindIII restriction enzyme sites for cloning. pMSCV-hygro plasmid was digested with PshAI and HindIII enzymes to remove the hygromycin resistance gene cassette, and the PCR-generated blasticidin gene cassette was cloned into these sites, generating pMSCV-blast. The ability of this vector to impart balsticidin-resistance was tested by transfecting the vector into 293T cells together with retroviral packaging vectors pHIT.60 and pMD.G. The retroviral supernatant was used to infect blasticidin-sensitive mouse embryonic fibroblasts. Transfected cells were grown

in medium containing balsticidin (5 ug/ml) and found to be blasticidin-resistant, whereas the mock-infected cells were fully sensitive. These results demonstrated the suitability of pMSCV-blast vector for mammalian cell transfection.

Cloning of wildtype and Cbl-independent EGFR and ErbB2, and wildtype Src, into retroviral vectors. Human ErbB2 cDNA in pCNA3 was obtained from Dr. Kermit Karraway (Harvard Medical School). A Quick-Change kit (Stratagene) was used to generate ErbB2-Y1112F mutant of this plasmid, as well as the Y1045F mutant of human EGFR in pCNA3 (previously established in the laboratory). The ErbB2-Y1112F and EGFR-Y1045F mutants represent Cbl TKB domain binding site mutants, which make them resistant to Cbl regulation [5, 6]. The wildtype and mutant EGFR and ErbB2 sequences were amplified using the PCR, and directionally cloned into the XhoI and HpaI sites of the respective pMSCV-puro and pMSCV-blast vectors. Mouse c-Src sequences were amplified using the PCR from a pLNCX construct provided by Dr. Joan Brugge (Harvard Medical School), and directionally cloned into the BglII and XhoI sites of pMSCV-hygro. The DNA sequences of all constructs were verified.

Establishment of the ability of retroviral constructs to direct the expression of expected proteins by transient transfection in 293T cells. As an initial test to determine if the various retroviral constructs direct the expression of respective proteins, the plasmids were transiently transfected into 293T cells and protein expression was assessed by immunoblotting. Anti-Src I.P. followed by anti-Src immunoblotting established the ability of pMSCV-hyg-cSrc to direct the over-expression of Src in 293T cells (Fig. 9). Similar analyses established the ability of pMSCV-puro- and pMSCV-blast-based ErbB2 (Fig. 10) and EGFR (Fig. 11) constructs to direct the over-expression of expected proteins in 293T cells.

Establishment of stable transfectant lines of 76N-TERT, 16A5 and M2-E6E7 cells over-expressing ErbB2. In order to test the ability of pMSCV-based vectors to direct overexpression of ErbB receptors in mammary epithelial cells, we carried out initial studies using the pMSCV-puro-ErbB2 construct. The pMSCV vector or pMSCV-ErbB2 construct was co-transfected with the packaging vector pIK into the packaging cell lineTSA54 and the supernatants were used to infect 16A5 and 76N-TERT cell lines. Following infection, the cells were selected in puromycin and resistant cell lines were obtained. Analysis of these cell lines at various passages was carried out by anti-ErbB2 immunoblotting of whole cell lysates (Fig. 12). It is clear that pMSCV-ErbB2 directs a high level of ErbB2 over-expression in both cell lines. These pooled populations (and their clones if required) will be used for further experiments. Importantly, these analyses provide a basis for the use of the retroviral infection approach for the overexpression of ErbB2, EGFR and Src individually and combinatorially (Task 3).

Overall, the studies carried out thus far have resulted in the identification of mammary epithelial cell lines suitable for our analyses, generation of retroviral constructs of EGFR, ErbB2 and Src, testing of the retroviral approach for ErbB overexpression, and establishment of pooled ErbB2 transfectants. Thus, both the reagents and techniques to proceed further with Tasks 3 to 7 are in place. These tasks will help test our hypothesis that Src regulates the intracellular traffic of ErbB receptor to a recycling endosomal signaling compartment.

Key Research Accomplishments:

- Characterized the EGFR, Cbl and Src expression in immortalized human mammary epithelial cells.
- Demonstrated that hTERT-immortalized human mammary epithelial cells are suitable for EGFR signaling studies.
- Developed retroviral vectors for expression of Src, EGFR and ErbB2.
- Generated ErbB2over-expressing transfectants of 16A5 and 76N-TERT mammary epithelial cell lines.

Reportable Outcomes:

Reagents:

- Generated a new pMSCV retroviral vector carrying blasticidin resistance marker for mammalian expression.
- Generated retroviral vectors encoding c-Src, EGFR (wild-type, EGFR (Y1045F), ErbB2 (wild-type) and ErbB2 (Y1112F).
- Generated ErbB2over-expressing transfectants of 16A5 and 76N-TERT mammary epithelial cell lines.

Conclusions:

In conclusion, our studies have led to identification of better mammary epithelial cell lines for analyses of ErbB signaling studies. We have also established an efficient retroviral infection approach for ErbB overexpression, and have constructed a series of retroviral constructs for overexpression of EGFR, ErbB2 and Src in human mammary epithelial cells. These results now allow us to proceed further with Tasks 3 to 7 to directly test our hypothesis that Src regulates the intracellular traffic of ErbB receptor to a recycling endosomal signaling compartment in mammary epithelial cells.

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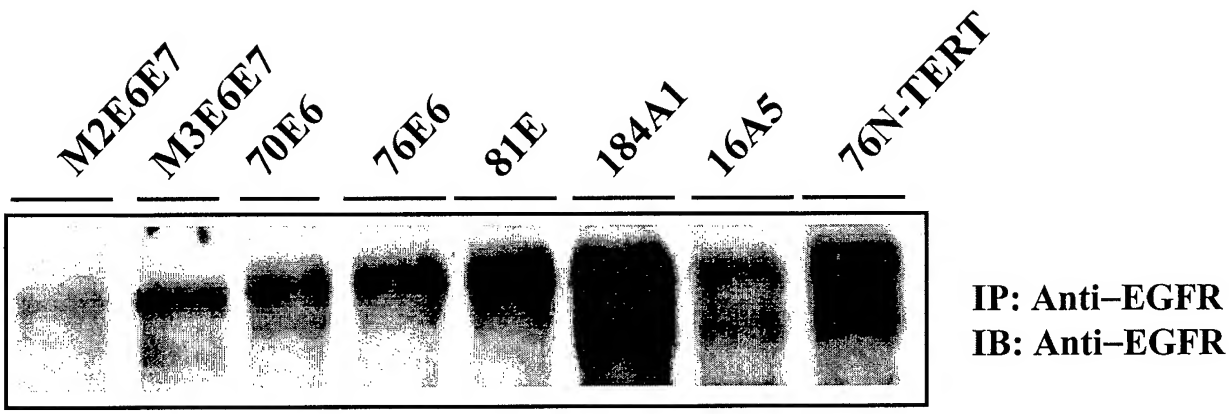

Figure 1. Expression of EGFR in different mammary epithelial cell lines.The indicated mammary epithelial cell lines were grown in DFCI-1 medium and cell lysates were prepared. 500μg aliquots of lysate protein were immunoprecipitated withan anti-EGFR antibody (clone 528).The immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane.The membrane was immunoblotted with a polyclonal anti-EGFR antibody (sc-03, Santa Cruz Biotechnology Inc.).

Cell Type	Control MFI	EGFR MFI
M2E6E7	2.7	31.0
16A5	1.7	37.5
76E6	3.4	38.5
76E6E7	2.7	38.5
81E6	3.2	38.8
70E6	4.4	44.1
76N-TERT	1.6	46.9
184A1	2.7	50.4

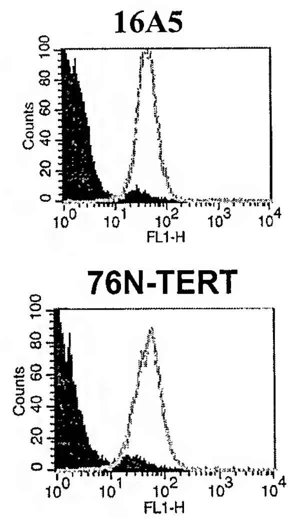

Figure 2. Various cell lines were stained with anti-EGFR antibody (528) or a control antibody (anti-Syk, 4D10) for 40 minutes on ice. Cells were then washed and stained with goat anti-mouse IgG-FITC conjugate (1:50 dilution, Biosource International) for 30 minutes on ice. Data were collected using a FACSort (BD) and analyzed using CellQuest software. Median fluorescence intensity (MFI) is depicted in the table for both control and anti-EGFR staining. FACS plots of representative cell lines (16A5 and 76N-TERT) are depicted to the right of the table.

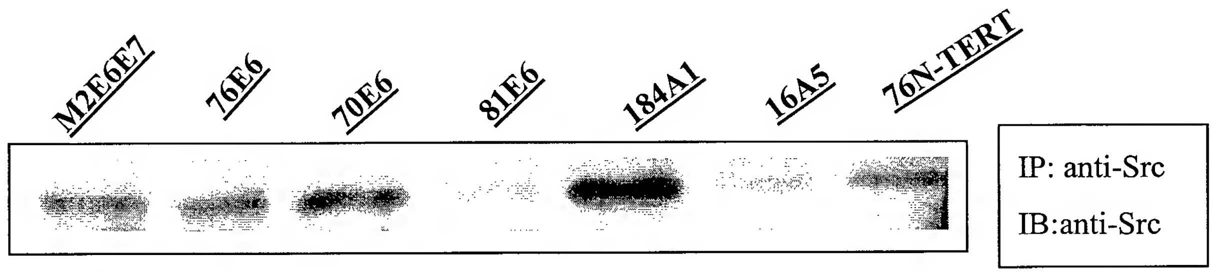

Figure 3. Expression of Src in different mammary epithelial cell lines. The indicated mammary epithelial cell lines were grown in DFCI-1 medium and cell lysates were prepared. 300 μg aliquots of lysate protein were immunoprecipitated with an anti-Src polyclonal antibody (Calbiochem). The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with the same anti-Src antibody used for immunoprecipitation.

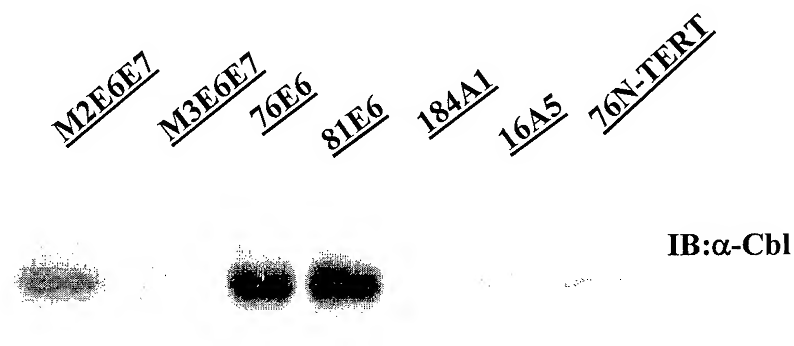

Figure 4. Expression of Cbl protein in different mammary epithelial cell-lines.The indicated mammary epithelial cell lines were grown in DFCI-1 medium and cell lysates were prepared. 50 μg of aliquots of lysate proteins were resolved by SDS-PAGE and transferred to a PVDF membrane.The membrane was immunoblotted with an anti-Cbl antiboby (C-15, Santa cruz Biotechnology Inc.).

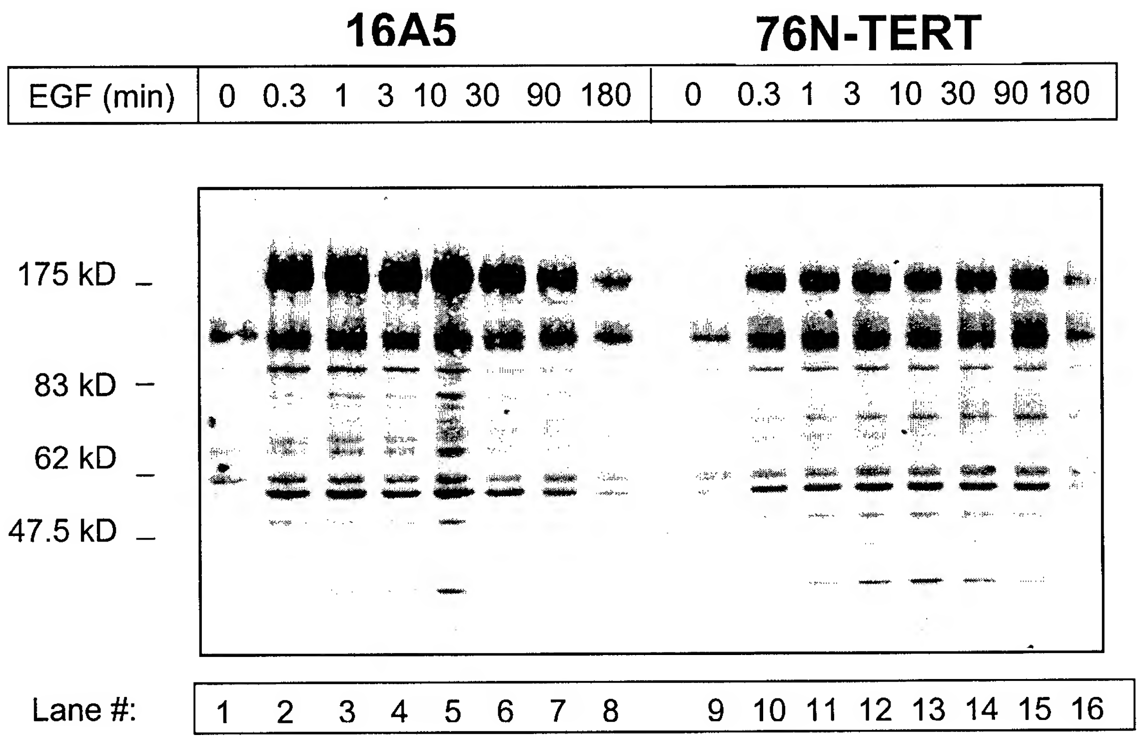

Fig. 5. Induction of protein tyrosine phosphorylation upon EGF treatment of 16A5 and 76N-TERT mammary epithelial cell lines. The cells were plated in DFCI-medium and then starved for 48 hours by growth in D3 medium. The cells were then left untreated (time 0) or treated with EGF (100 mg/ml) for 0.3, 1, 3, 10, 30, 90 or 180 min, and cell lysates were prepared. Equal aliquots (50μg) of lysate protein from each sample were resolved by SDS PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with anti-Tyr (P) antibody 4G10.

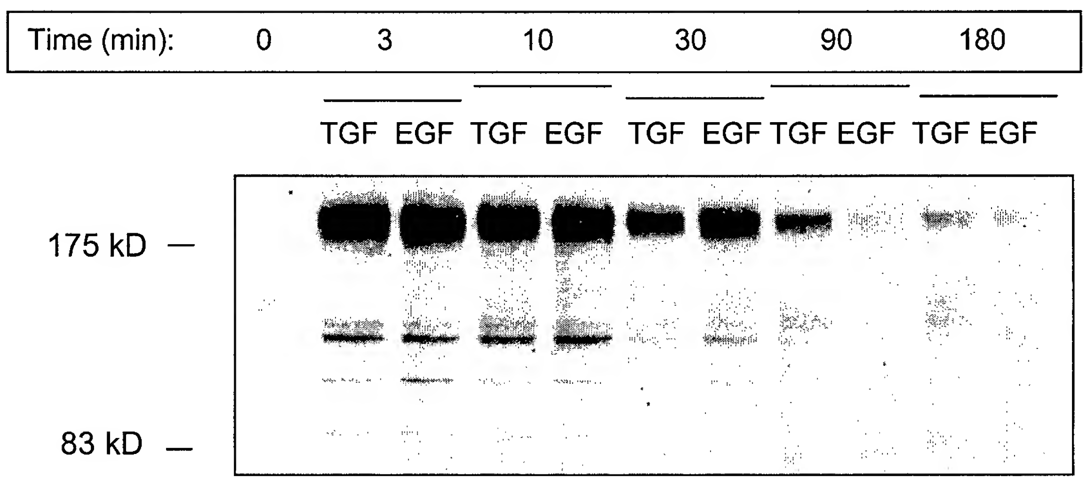

Fig. 6. Protein tyrosine phosphorylation induced by TGF-alpha or EGF treatment of 76N-TERT mammary epithelial cells. The cells were plated in DFCI-1 medium and then starved of EGF by growth in D3 medium for 48 hours. The cells were then left untreated (time 0) or treated with TGF alpha or EGF (40 ng/ml) for 3, 10, 30, 90 and 180 min. Cell lysates were prepared and equal aliquotss (50 μg) of lysate protein from each sample were resolved by SDS PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with anti-Tyr (P) antibody 4G10.

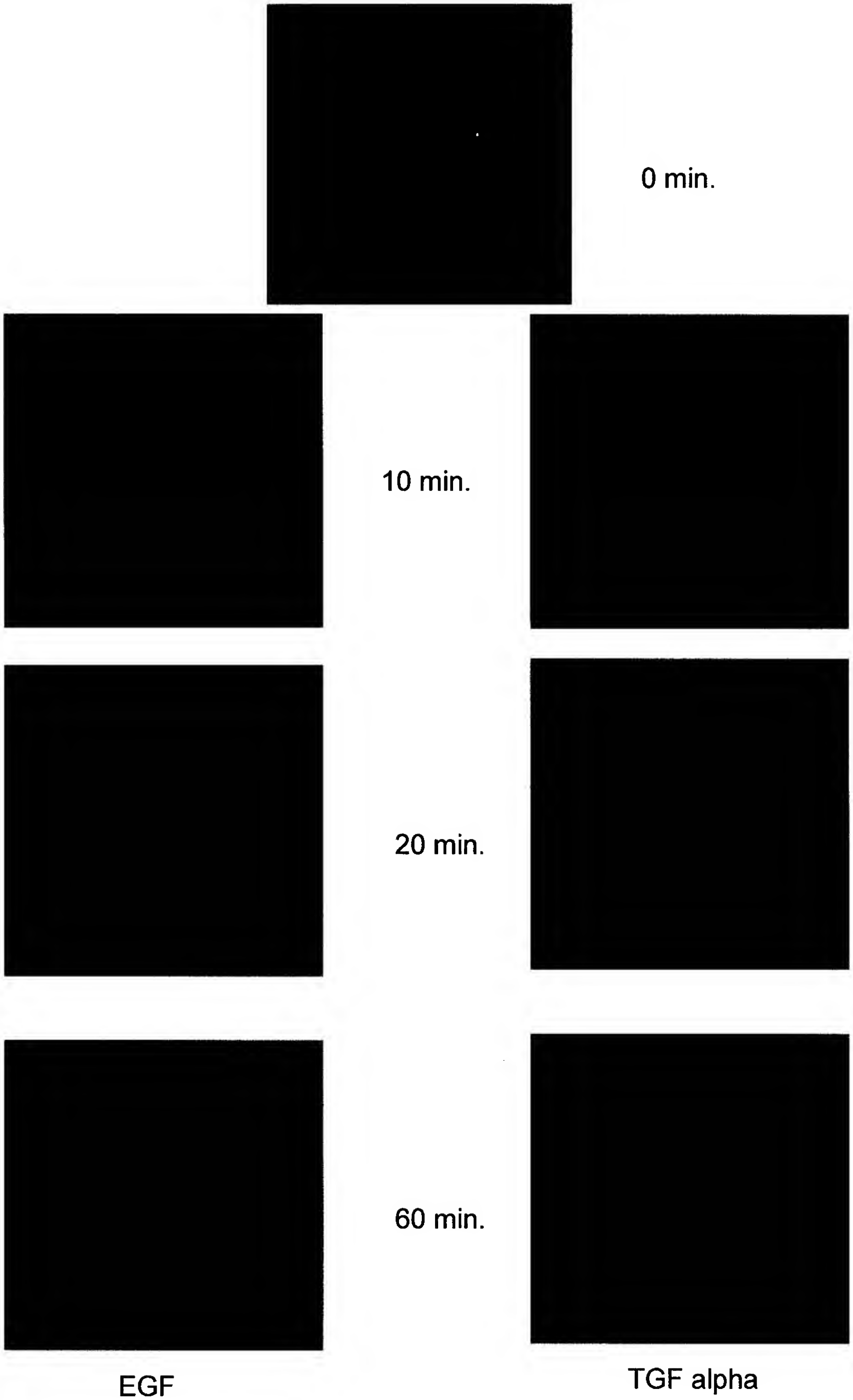

Fig. 7. Localisation of EGFR in intracellular vesicles following stimulation of 76N-TERT cell swith EGF or TGF alpha. 76N-TERT cells were grown on coverslips in DFCI-1 mediun, and then starved of EGF by growth in D3 medium for 24 hours. The cells were either left untreated (time 0) or treated with EGF or TGF alpha (40 ng/ml) for 10, 20 or 60 min. The cells were fixed with 3.7% paraformaldehyde in PBS for 15 min. and treated with saponin, followed by immunostaining with anti-EGFR antibody 528 and fluorescence microscopy.

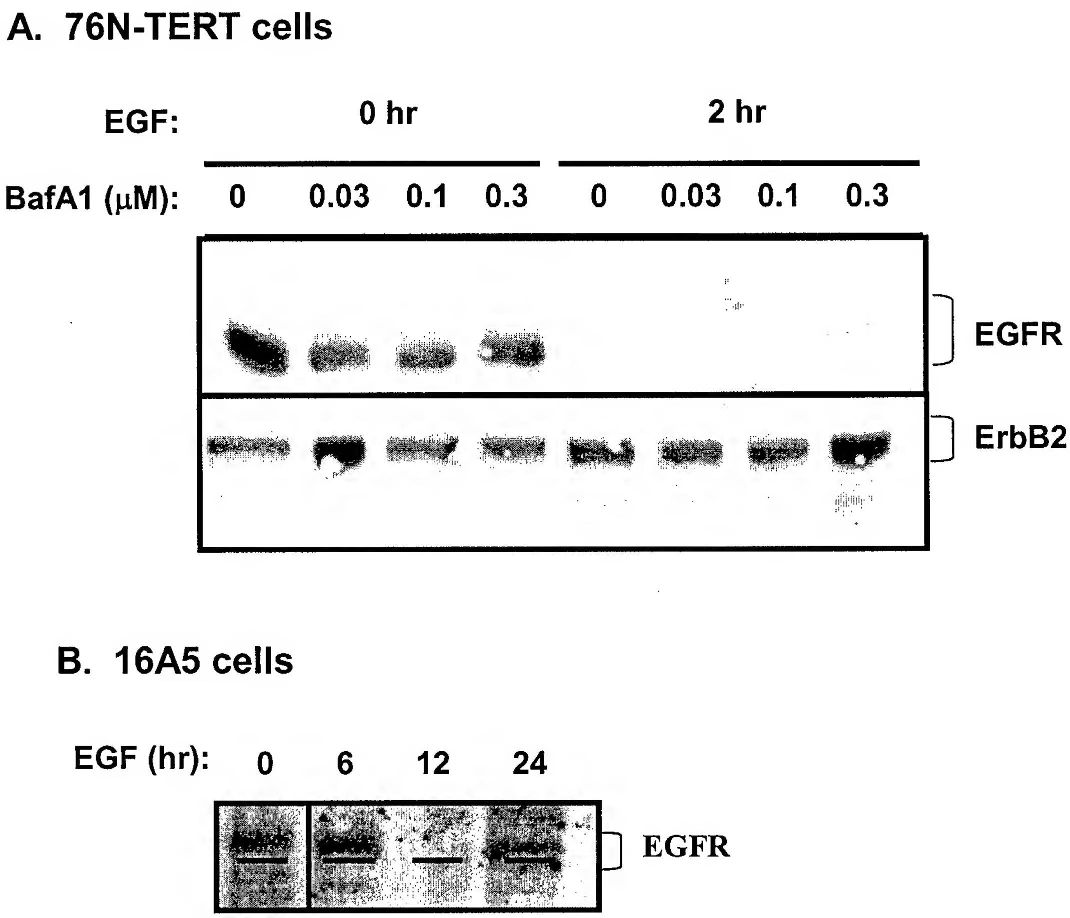

Figure 8. Ligand-induced EGFR down-regulation in 76N-TERT and 16A5 mammary epithelial cell lines. (A) 76N-TERT cells were plated in DFCI-1 medium followed by EGF deprivation for 48 hours in D3 medium prior to EGF stimulation. The lysosomal inhibitor bafilomycin A1 was added to the cells at the indicated concentrations one hour before EGF stimulation. 50 μg aliquots of lysate protein were resolved by SD-PAGE and immunoblotted with anti-EGFR and antibodies. Note the essentially complete loss of EGFR signal at 2 h after EGF treatment in the absence of bafilomycin A1, while no change in ErbB2 signal is seen. The loss of EGFR signal is partially reversed by Bafilomycine A1. (B) 16A5 cells were plated in DFCI-1 medium and deprived of EGF for 24 hours in D3 medium prior to EGF stimulation. Shown is an EGFR western blot performed on 50μg of whole cell lysate. Note that relatively little change in EGFR signal is seen at 6h post-EGF treatment.

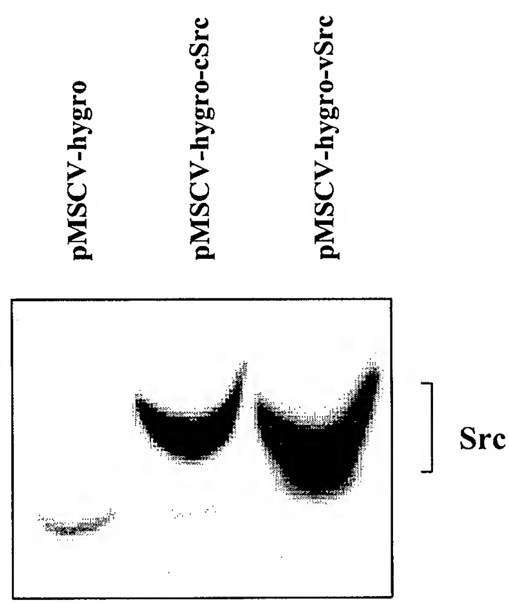

Fig 9. The retroviral constructs of c-Src and v-Src encode immunoreactive proteins upon transient transfection into 293T cells. 293T cells were transfected with pMSCV-hygro vector or the indicated c-Src or v-Src construct DNA and cell lysates were prepared after 48h. Equal aliquots (50 ug) of lysate protein were resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with an anti Src polyclonal antibody (Calbiochem).

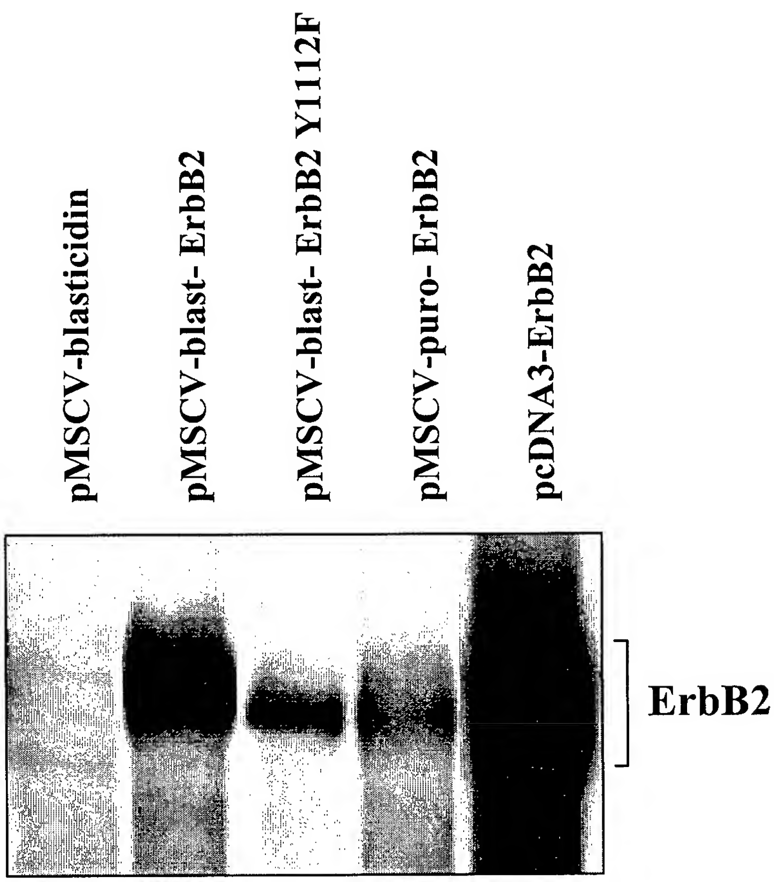

Fig 10. The retroviral constructs of wild type ErbB2 and ErbB2-Y1112F mutant encode immunoreactive proteins following transient transfection into 293T cells. 293T cells were transfected with pMSCV-blasticidin vector or the indicated constructs. As a positive control, the cells were transfected with pCNA3-ErbB2 construct (last lane). Cell lysates were prepared after 48h and equal aliquots (50 ug) of lysate protein were resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with an anti ErbB2 polyclonal antibody (Neu-C18, Santa Cruz Biotechnology).

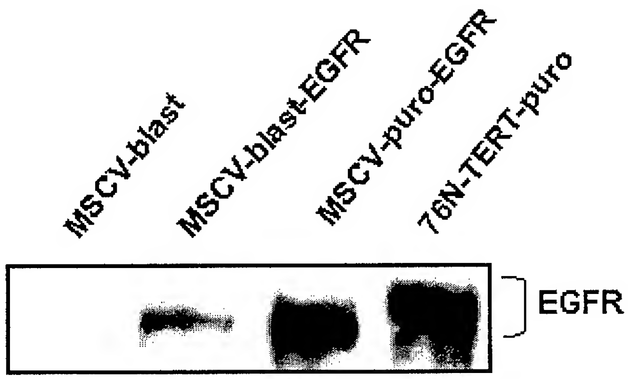

Figure 11. The ability of pMSCV-based retroviral constructs to encode immunoreactive EGFR upon transfection in 293T cells. 293T cells were transfected with 10 ug each of pMSCV-blast vector, pMSCV-blast-EGFR or pMSCV-puro-EGFR constructs, using the calcium phosphate method. Total cell lysates were prepared 48 hours post-transfection and 50μg aliquots of the lysate protein were run on an SDS-PAGE gel and immunoblotted with an anti-EGFR rabbit polyclonal antibody (sc-003, Santa Cruz Biotechnology). The 76N-TERT-puro lysate was used as a positive control.

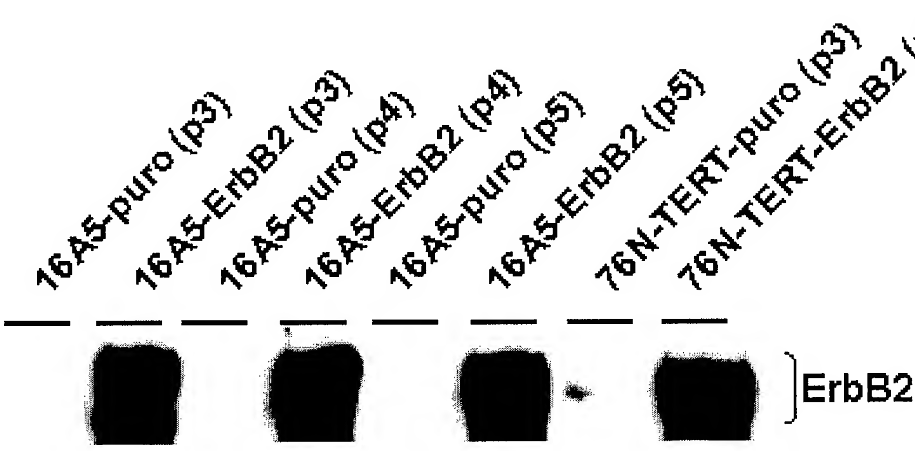

Figure 12. Retrovirus-mediated over-expression of ErbB2 in 16A5 and 76N-TERT mammary epithelial cell lines. The packaging cell line (tsa54) was cotransfected with the pMSCV-puro vector or the pMSCV-puro-ErbB2 construct together with the packaging plasmid pIK, using the calcium phosphate method. Retrovirus-containing supernatant was collected 24 hours after transfection, centrifuged to remove cell debris, and stored at –70°C. Retroviral supernatants were subsequently quick-thawed at 37°C and used to infect 16A5 and 76N-TERT cells for three sequential four-hour infections in the presence of polybrene (4μg/ml). Virally transduced cells were selected in 0.5μg/ml puromycin for 5 days. 50μg aliquots of lysate protein were resolved by SDS-PAGE and subjected to anti-ErbB2 immunoblot analysis. Passage numbers in parentheses next to cell line designations represent the number of passages post-transfection. Note high level of ErbB2 overexpression in cells transduced with ErbB2 retrovirus compared to the level of endogenous ErbB2 in cells transduced with the vector alone.